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Evaluation of *in vitro* biological activities: antioxidant; anti-inflammatory; anti-cholinesterase; anti- xanthine oxidase, anti-superoxyde dismutase, anti-α-glucosidase and cytotoxic of 19 bioflavonoids

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Abstract: Pure compounds belonging to phenolic family were studied for their biological potential such as 5,8-dihydroxy-1,4-naphthoquinone (M1), rutin hydrate (M2), 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (M3), taxifolin (M4), myricetin (M5), plumbagin (M6), silibinin (M7), dihydromyricetin (M8), shikonin (M9), quercetin 3-β-D-glucoside (M10), (±)-taxifolin hydrate (M11), cardamonin (M12),(−)-epicatechin (M13), 9-chloro-10-hydroxy-anthracene-1,4-dione (M14), 9-chloro-10-hydroxy-2,3-dimethyl-anthracene-1,4-dione (M15), 2-chloro-3-(2-hydroxy-5-methylanilino)-1,4-naphthoquinone (M16), 2-chloro-3-(4-hydroxy-phenylamino)-(1,4) naphthoquinone (M17), 2-chloro-3-(3,5-di-tert-butyl-4-hydroxy-phenyl)-(1,4)-naphtoquinone (M18), and myricitrin dihydrate (M19). These molecules were chosen based on two reasons; the limited or total absence of their exploitation in several studied activities and the use of other tests for the same activity. The evaluation of the *in vitro* anti-acetyl-cholinesterase (AChE), anti-5-lipoxygenase (5-LOX), anti-xanthine oxidase (XOD), anti-alpha glucosidase, anti-superoxide dismutase (SOD), anti-oxidant (DPPH (1, 1-diphenyl-2-picrylhydrazyl) and ABTS (2, 2- azinobis-3-ethylbenzothiazoline-6-sulphonate)), and anticancer activities of mentioned 19 molecules was explored during this work. M3, M14, M15, M16, M17, M18, M19 were exploited for the first time for such purposes. Tested compounds were shown to have interesting radical scavenging abilities against DPPH radicals, and the highest molecules among them were M19 and M5 (IC_{50} = 12.0 and 15.5 µM, respectively), and M4, M19 and M2 against ABTS (IC_{50}= 1.9, 4.3 and 4.3 µM, respectively). Moreover, the majority of products showed very important cytotoxic activity since IC_{50} values were ranging between (IC_{50}= 0.2 µM (M1) and 79 µM (M8)) against HCT116 cell line, and values of IC_{50}= 0.2 µM for M1 against MCF7 cell line. All new molecules (non studied before) were shown to have great cytotoxic effect against both cancer cell lines. Furthermore, molecule M5 was shown to have anti-inflammatory potential via the inhibition of 5-LOX enzyme (65% at 100 µM). In addition, M19 showed important anti XOD activity with 47% of inhibition at 100 µM. Also, it has been found that compound M3 had the best anti alpha glucosidase activity with 43.8 % of inhibition at 100 µM, the highest anti-AChE effect (IC_{50}= 14.5 µM), and the best effect towards SOD (IC_{50}= 10.0 µM). A structure-activity relationship study was also performed.

Key words: Bioflavonoids; Antioxidant; Anti-inflammatory; Anti-cholinesterase; Anti-XOD; Anti-SOD; Anti-α-glucosidase; Anticancer (MCF7 and HCT116).

Introduction

Natural substances particularly from plants have always been known for their medicinal properties (1). They are an inexhaustible source of chemical molecules and an interesting source of compounds with pharmacological activities. Polyphenols, plant secondary metabolites, can be classified depending on their chemical structures into phenolic acids, flavonoids, stilbenes, and lignin (1), with an enormous variety of physiological functions in plants (2). Recently, researches has been concentrated on the intervention of polyphenols in the control of a wide range of enzymes in cells (3).

Although natural substances that possess biological activities have been the subject of numerous investigations and a wide variety of plants has been screened, it remains interesting to work on non- studied molecules for all activities.

In the biological system, many enzymes are known to play a big role in dysfunction of the system and the appearance of diseases like oxidative stress and inflammation, which if it persists lead to the generation of Alzheimer, diabetes, gout and cancer. Therefore, inhibition of these enzymes become the target for therapeutic treatment of multiple dysfunctions and serious health problems.

Most often, a desired biological response is not due to the whole plant but the presence of one bioactive components. The difficulty in extracting active ingredient from a plant in one hand, and its low productivity on the other hand promotes us to resort the use of pure compounds. In this work, the *in vitro* biological activities; anti-cancer (breast cancer and colon cancer), anti-inflammatory, anti- acetyl cholinesterase, anti-α-glucosidase, antioxidant, anti-Xanthine oxidase (XOD), and anti-superoxide dismutase (SOD) of 19 natural phe-

nolics will be performed. Phenolic compounds were chosen starting from molecules with common phenolic base that are non-studied by previous investigators or studied in other activities. Several natural molecules will be evaluated for the first time against the diseases mentioned above.

Materials and Methods

Chemicals

All chemicals used were of analytical reagent grade. They were purchased from Sigma-Aldrich-Fluka (Saint-Quentin France) and stored under the optimum conditions indicated by the manufacturer. The 5,8-dihydroxy-1,4-naphthoquinone, rutin hydrate, 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone, taxifolin, myricetin, plumbagin from *Plumbago indica*, silibinin, dihydromyricetin, shikonin, quercetin 3-β-D-glucoside, (±)-taxifolin hydrate, cardamonin, (-)-epicatechin, 9-chloro-10-hydroxy-anthracene-1,4-dione, 9-chloro-10-hydroxy-2,3-dimethyl-anthracene-1,4-dione,2-chloro-3-(2-hydroxy-5-methylanilino)-1,4-naphthoquinone,2-chloro-3-(4-hydroxy-phenylamino)-(1,4)-naphthoquinone, 2-chloro-3-(3,5-di-tert-butyl-4-hydroxy-phenyl)-(1,4) naphthoquinone, myricitrin dihydrate, pyrogallol, DPPH, ABTS, Ascorbic acid, potassium persulfate, NaH_2PO_4, Na_2HPO_4, NaCl, 5-lipoxygenase, arachidonic acid, linoleic acid, sodium phosphate buffer, DMSO, Nordihydroguaiaretic acid (NDGA), acetyl cholinesterase solution, DTNB, acetylthiocholine iodide, galanthamine, MTT solution, tamoxifen, α-glucosidase solution, p-nitrophenolfrom p-nitrophenyl α-D-glucopyranoside, xanthine oxidase solution, allopurinol, superoxide dismutase solution, acarbose was from Vidal.

Methods

Free radical scavenging activity DPPH test

Antioxidant scavenging activity was studied using the 1, 1-diphenyl-2-picrylhydrazyl free radical (DPPH) with some modifications (4). About 20 µL of various dilutions of each sample was mixed with a 0.2 mM methanolic DPPH solution. After 30 min of incubation at 25 °C, the absorbance at 524 nm was recorded as $A_{(sample)}$. For the $A_{(blank)}$, the same experimentation was applied for a solution devoid of the test material and then, the absorbance was recorded. Then, for each solution the free radical scavenging activity was calculated as percent inhibition as the following equation:

$$\% \text{ Inhibition} = 100 \times ((A_{(blank)} - A_{(sample)}) / A_{(blank)}).$$

The IC_{50} is the concentration required for the test material to cause a 50% decrease in DPPH concentration. Ascorbic acid was used as reference. All the measurements were performed in triplicate.

ABTS radical-scavenging test

The radical scavenging capacity for 2, 2- azinobis-3-ethylbenzothiazoline-6-sulphonate (ABTS) radical cation of the test samples was determined with some modifications (4). Briefly, a solution of ABTS (7 mM) was mixed with a solution of $K_2S_2O_8$ (2.5 mM), followed by a storage for 16 hours in the dark at room temperature. The mixture was then, diluted with water and the absorbance was determined at 734 nm. A volume of 20 µL of ABTS diluted was added to each sample. Ascorbic acid was used as reference. The capacity of free radical scavenging was expressed by IC_{50} (µM), which indicate the required concentration to scavenge 50% of ABTS radicals. The same equation described previously for the DPPH assay was used to calculate this capacity. All measurements were performed in triplicate.

Anti-inflammatory activity

The anti-inflammatory activity of pure compounds was determined on Soybean lipoxygenase (5). The presence of linoleic acid, as a substrate, the 5-lipoxygenase enzyme, catalyzes the oxidation of unsaturated fatty acids containing structures of 1-4 pentadienes, then the substrate is oxidized to a conjugate diene via the action of the enzyme. Practically, 20 µL of compounds was mixed with 150 µL sodium phosphate buffer (pH 7.4), 20 µL of 5-lipoxygenase and 60 µL of linoleic acid (3.5 mM), to finally obtain a volume of 250 µL. However the substrate was replaced by 30 µL of buffer solution for the blank. Then, the mixture was incubated at 25 °C for 10 min and the absorbance was determined at 234 nm. Nordihydroguaiaretic acid (NDGA) was used as reference. The following equation allows to calculate the inhibition:

$$I (\%) = ((A_{control} - A_{sample}) / A_{control}) \times 100$$

A_{sample} is the absorbance of the extract containing reaction and $A_{control}$ is the absorbance of reaction control. Tests were carried out in triplicate. The IC_{50} value is the concentration of the extract that caused 50% enzyme inhibition.

Anti-cholinesterase activity

The enzymatic activity was measured using Ellman's method (6) as previously reported (7). A solution of 50 µL sodium buffer (0.1 mM at pH 8), was mixed with 25 µL of AChE solution, 25 µL of extract and 125 µl of DTNB were added in a 96-well microplate and incubated for 15 min at 25 °C with stirring. All extracts were re-suspended in the DMSO followed by dilution in the buffer so that the DMSO does not exceed 1%. The addition of 25 µL of acetylthiocholine iodide initiated the reaction. After 10 min of incubation, the hydrolysis of ACTHi was detected by the formation of the 5-thio-2-nitrobenzoate anion as mentioned below, and the absorbance was determined at 412 nm. The IC_{50} value is the concentration of the extract that caused 50% enzyme inhibition. Galanthamine was used as reference. All the measurements were performed in triplicate.

Anti-α-glucosidase activity

The α-glucosidase inhibitory activity was determined (5). Briefly, a mixture of α-glucosidase solution (1U/mL) and the test compound was incubated for 10 min at 25 °C, then, the substrate p-nitrophenyl α-D-glucopyranoside (5 mM) was added in the phosphate buffer (0.1 mM) to the mixture to let the reaction begin. After a second incubation for 5 min at 25 °C, the absorbance was detected at 405 nm. Acarbose was used as reference. All the measurements were performed in triplicate.

Anti-xanthine oxidase activity

In order to evaluate the potential anti-hyperuricemia,

the assays were conducted to investigate whether various concentrations of tested compounds inhibited the catalytic activity of XOD and hence bloc the generation of uric acid. The protocol described by Lin et al. (8) was used with some modifications (7). The tested compounds were dissolved in DMSO and diluted to a final concentration containing less than 1% DMSO. The assay consists of mixing 50 µL of test compound, 60 µL of phosphate buffer (70 mM at pH 7.5) and 30 µL enzyme solution (0.1 U/mL). After an agitation and an incubation for 15 min at 25 °C, the addition of 60 µL of substrate (xanthine 150 µM) initiated the reaction. The blank solution was similar to test sample except the presence of enzyme solution. Then, a second incubation and agitation was established for 5 min at 25 °C and the absorbance was determined at 295 nm. Allopurinol was used as positive control. The IC_{50} values of the samples were calculated from regression lines of a plot of the percentage of inhibition on XOD activity according to the concentrations of the samples. The percentage of inhibition was calculated as mentioned in the equation below:

$$\% \text{ Inhibition} = 100 - (A_{sample}/A_{control}) \times 100$$

A_{sample} and $A_{control}$ are absorbance for the sample and the blank, respectively. Each test was repeated in triplicate.

Anti-superoxide dismutase activity

SOD (Mn SOD) is an enzyme with antioxidant activity which inhibits the pyrogallol's autoxidation, thus, when it is inhibited by a molecule, the absorbance of the formed product after the autoxidation of pyrogallol is high (9). The principle of this method consists on the competition on the superoxide anion between its implication in the autoxidation of pyrogallol or its dismutation by SOD as previously described (7). Briefly, the assay mixture consisted of 50 µL test solution of compound and 120 µL SOD solution (0.1 Unit/mL). The same for the control solution which was prepared by mixing 50 µL of test compound and 120 µL of 50 mM buffer trizma/diethylenetriaminepenta acetic acid (DTPA), while the blank control contains just the DMSO and buffer. The samples were mixed and incubated for 4 min at room temperature. The reaction was initiated by the addition of pyrogallol (30 mM) and then stirred and incubated for the second time for 5 min at 25 °C, at that moment, the absorbance was immediately measured at 325 nm. After that, it was read every single minute for 4 min successively. Control samples were prepared identically but without the extracts. The percentage of inhibition was calculated as below:

$$\% \text{ Inhibition} = \frac{\text{average } (\Delta DO_{(sample)}) - \text{average } (\Delta DO_{(blank\ sample)})}{\text{average } (\Delta DO_{(control)}) - \text{average } (\Delta DO_{(sample)})} \times 100$$

Cell viability evaluation

The cell viability was evaluated by MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay. Briefly, MTT is a yellow tetrazolium salt. In the biological system, the succinate deshydrogenase, a mitochondrial enzyme of the viable cells, reduces the tetrazolium to dark blue formazan precipitate. The quantity of this product is proportional to the quantity of active cells. The anticancer activities of each compound was determined via the human cancer cell line HCT-116 for colorectal cancer cells and MCF-7 for breast cancer cells (5). Cells were distributed in 96- well plates at 4 x 10^4 cells/well in 100 µL, and then 100 µL of a culture medium containing compounds at various concentrations were added. The mitochondrial reduction of MTT to formazan was explored to determine the cytotoxic effect of those compounds. Tested compounds were suspended in the DMSO and then diluted, so the concentration of DMSO does not exceed 1% in the mixture. Tamoxifen was used as a positive control. The test was performed in triplicate.

Statistical Analysis

All data were expressed as means ± standard deviation of triplicate measurements. The confidence limits were set at $p < 0.05$. Correlations were carried out using the correlation and regression in the Microsoft® EXCEL program.

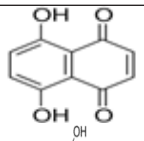
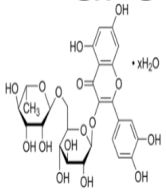
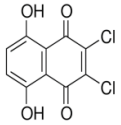
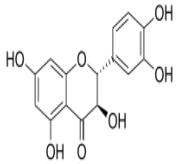
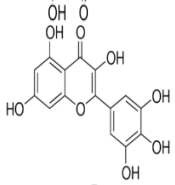
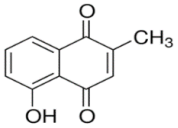
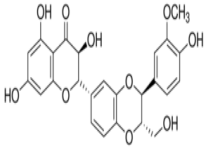
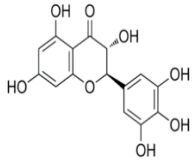
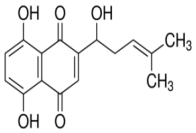
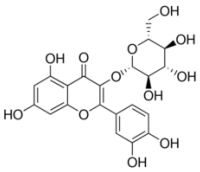
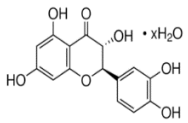
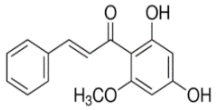
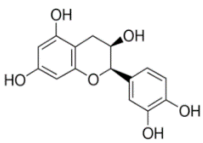
Results and Discussion

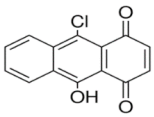
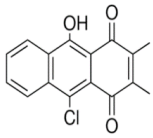
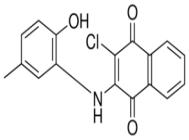
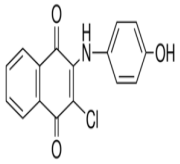
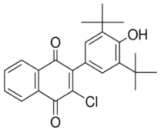
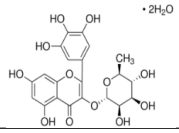
The free radical-scavenging capacity of tested compounds was evaluated with the DPPH and ABTS assays as represented in Table 1, within tested molecules, M14, M15, M16, M17, M18 and M19 have never been tested for their antioxidant potential by previous investigators. During this work, the majority of selected molecules has shown antioxidant ability through the DPPH assay. Since, the hierarchy between them for their antioxidant capacity against DPPH radicals is: M19 < M5 < M2 < M13 < M8 < M4 < M10 < M17 < M11 with IC_{50} values as follows: 12.0 ± 0.1; 15.5 ± 0.5; 16.9 ± 0.7; 29.1 ± 0.7; 29.7 ± 0.3; 35.3 ± 1.6; 35.9 ± 0.1; 39.4 ± 0.2 and 50.2 ± 0.2 µM, respectively (Table 1).

On the other hand, the capacity of tested samples to scavenge free radicals was determined by another method based on decolorization of the solution of ABTS°+ cation, at a specific absorbance (734 nm) for a precise time of incubation. In the present study, IC_{50} values varied between 1.9 ± 0.1 µM and 40.7 ± 1.1 µM. The hierarchy between tested pure compounds for antioxidant capacity using this method was M13 < M19 < M2 < M4 < M10 < M5 < M11 < M8 < M7 < M1 < M3 < M9 < M17 < M12 at a concentration of 100 µM (Table 1). Those values are comparable to vitamin C (1.0 µM).

This antioxidant potential is mostly due to their chemical structure. Briefly, the antioxidant activity of M19 (Table 1) was the highest to scavenge DPPH radical, with an IC_{50} equivalent to 12.0 ± 0.1 µM, followed by M5 (IC_{50} equal to 15.5 ± 0.5 µM) compared to those of other investigators, who gave different results for M5 from found data (with 96% inhibition at 100 µM). Indeed, Wang et al. (10) reported a percentage of inhibition about 54% at 31.4 µM, and Masuoka et al. (11) found a value of 30% at 5 µM using DPPH method for same molecule. Whereas, for M2, Chua (12), have found a percentage of inhibition of DPPH radicals equal to 90.4% at a concentration of 82 µM which is very close to found results (93% at 100 µM) as mentioned on Table 1. While, Latté and Kolodziej (13) and Masuoka et al. (11), have noted for the same molecule, using the same test, higher value, since, IC_{50} equal to 9.1 µM and 5.8 µM, respectively. Otherwise, for the M4, which reg-

Table 1. Antioxidant and anticancer activities of exploited compounds.

Founded results		Antioxidant activity		Cytotoxic activity	
Compounds	**Chemical structure**	**DPPH IC_{50} (µM)**	**ABTS IC_{50} (µM)**	**MCF7 (IC_{50} µM)**	**HCT116 (IC_{50} µM)**
M1:5,8-dihydroxy-1,4-naphthoquinone		>100.0	8±0.0	0.2±0.0	0.2±0.0
M2 : Rutin hydrate		16.9±0.6	4.3±0.6	>100.0	>100.0
M3 : 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone		>100.0	9.8±0.3	3.6±0.3	0.3±0.0
M4: Taxifolin		35.3±1.6	4.9±0.1	>100.0	>100.0
M5 : Myricetin		15.5±0.5	5.7±0.2	42.0±4.0	28.0±3.0
M6 : Plumbagin from *Plumbago indica*		>100.0	>100.0	2.5±0.4	27.0±4.0
M7 : Silibinin		>100.0	6.3±0.6	30.0±0.0	>50.0
M8 : Dihydromyricetin		29.7±0.3	5.9±0.1	37.0±5.0	79.0±7.0
M9 : Shikonin		>100.0	11.8±0.4	0.6±0.1	0.4±0.0
M10 : Quercetin 3-β-D-glucoside		35.9±0.1	5.0±0.3	>100.0	74.0±6.0
M11 : (±)-Taxifolin hydrate		50.2±0.2	5.8±0.3	>100.0	>100.0
M12 : Cardamonin		>100.0	40.7±1.1	30.0±3.0	32.0±5.0
M13:(−)Epicatechin		29.1±0.7	1.9±0.1	>100.0	>100.0

M14 : 9-Chloro-10-hydroxy-anthracene-1,4-dione		>100.0	>100.0	2.7±0.3	1.5±0.2
M15 : 9-Chloro-10-hydroxy-2,3-dimethyl-anthracene-1,4-dione		>100.0	>100.0	>100.0	>100.0
M16 : 2-Chloro-3-(2-hydroxy-5-methylanilino)-1,4-naphthoquinone		>100.0	>100.0	0.9±0.1	17.0±2.0
M17 : 2-Chloro-3-(4-hydroxy-phenylamino)-(1,4) naphthoquinone		39.4±0.2	17.3±1.1	2.9±0.3	9.0±1.0
M18 : 2-Chloro-3-(3,5-di-tert-butyl-4-hydroxy-phenyl)-(1,4)-naphthoquinone		>100.0	>100.0	16.0±2.0	14.0±1.0
M19 : Myricitrin dihydrate		12±0.1	4.1±0.1	40.0±3.0	>100.0

istered an interesting antioxidant activity against DPPH radicals (IC_{50} = 35.0 µM) and an inhibition of 95.7% at 100 µM. Choi et al. (14) and Krishnan et al. (15) have found various antioxidant abilities using DPPH assay, since IC_{50} equivalent to 54.2 µM and 16.5 µM, respectively. For the M8, Wu et al. (16), have reported a higher inhibition percentage against DPPH radicals, about 73% at 62.5 µM, than those found with 95% at 100 µM. For M7, Zarelli et al (17), reported an IC_{50} equal to 392 µM

Furthermore, concerning M10, Beara et al. (18) and Silva et al. (19) have found results, which are in line with established data via the same test with an IC_{50} equivalent to 23.8 µM and 25.4 µM, respectively. In addition, Choi et al. (14), have shown an identical results to what was found in this work, through the DPPH test, Whereas, other investigators like Latté and Kolodziej (13), Masuoka et al. (11), and Silva et al. (20) have reported different antioxidant activity via the DPPH assay, IC_{50} equal to 14.6, 6.5 and 11.7 µM, respectively. While Nile and Park have reported less important data than those found in this work, since for the same molecule they reported an IC50 equal to 128.5 µM (21). For M13, with an IC_{50} equal to 29 µM. Jung et al. (22), have registered an IC_{50} via the DPPH test, four fold more important than obtained data (6.4 µM).

In this study, the ABTS assay was also explored to better understand the scavenging capacities for tested compounds, likewise, many researchers have used the same test to confirm the antioxidant potential, Krishnan et al. (15) have found for M4 an IC_{50} equal to 218 µM, which is so weak compared to obtained result (4.9 µM). In addition, for the M10, Compaoré et al. (23), have shown an IC_{50} equal to 11.8 µM which is twice less important than what was found in this work, with a value of 5 µM. While for M9, it is reported by Han et al. (24), who have found a percentage of inhibition of 95% at 277.5 µM, which is less interesting than results found by us (99% at 100 µM) (Table 1). Tested compounds (M1, M2, M3, M4, M5, M7, M8, M9, M10, M11, M12 and M13) were excellent scavengers against ABTS cation with IC_{50} = 8 ± 0; 4.3 ± 0.6; 9.8 ± 0.3; 4.9 ± 0.1; 5.7 ± 0.2; 6.3 ± 0.6; 5.9 ± 0.1;11.8 ± 0.4; 5.0 ± 0.3; 5.8 ± 0.3; 40.7 ± 1.1 and 1.9 ± 0.1 µM respectively (Table 1), but presented variable scavenging abilities against DPPH as mentioned on Table 1. It should be notified that M19 has the most potent antioxidant capacity as it has the more interesting IC_{50} through both tests, since IC_{50} = 4.1 ± 0.1 and 12 ± 0.1 µM, against ABTS and DPPH radicals, respectively. The ABTS test is marked by higher repeatability than the DPPH test. This difference could be due to the kinetic constants of reactions, which is generally higher between ABTS and bioflavonoids than that for DPPH and bioflavonoids. Further, within those products, M1, M3, M7, M9 and M12 have shown an interesting scavenging potential against ABTS but not DPPH radicals, that might depend on the target ABTS which is less cumbersome compared with DPPH. Moreover, the chemical structure of tested compounds could be involved, for example for M9 shikonin, the presence of 1' position of hydroxyl group (OH) makes it readily undergo reactions, to stabilize the free radical of shikonin which may be responsible for the high ABTS scavenging activity, and hence, can exert antioxidant activity. It is generally accepted that phenolic compounds are potential antioxidant known for their ability to donate hydrogen atoms. In addition, the ortho-dihydroxy phenolic moiety as it is the case for (M1, M2, M3, M4, M5, M7, M8, M9, M10, M11, M12, M13 and M19) make it more soft to donate hydrogen atoms to active free radicals to form the neutralized phenoxyl radical of the compound (25). It has been focused on the link between the antioxidant activity of bioflavonoids as hydrogen donating free radical scavengers and their chemical structure. In fact, the presence of the –CH=CH-COOH group in the hydroxylated cinnamates guarantees the ability of H- donating following by radical stabilization, than the

carboxylate group in the hydroxyl benzoate (26).

Anti-inflammatory activity

The 5-LOX inhibition by natural molecules was reported as shows Table 2. During this investigation, some molecules, which have never been exploited for the evaluation of this enzyme inhibition were exploited such as M14, M15, M16, M17, M18 and M19. Compounds showed moderate values of enzymatic inhibition. They are not very active via this method. Similarly, there was a correlation between obtained results in this work and earlier investigators'. Since, Ahmad et al. (27), who have not found a significant inhibition of LOX for M12 (less than 50% at different dilutions starting by 50 µM), therefore they have used another method involved cellular models. Actually, they have reported anti-inflammatory proprieties of M12 as an inhibitor of NO (16.1 µM) and TNF-α (4.6 µM), via inhibition of NF-κB pathway. In the same context, Wang et al. (28) have used another method to evaluate the anti-inflammatory activity for M4, via the ROS production. For M2, Li et al, reported an IC_{50} equal to 261.9 µM (29), and for M3, Saffoon et al, have found an IC_{50} higher than 386.0 (30).

Concerning M5, Kawabata et al. (31), found a value equivalent to 30 µM via the NO production assay. Also, Li et al. (32) have reported that M5 is a potent inhibitor of COX-2, with an IC_{50} equal to 222.5 µM. For M5, literature reported variable values of IC_{50} ranging between 30 and 222 µM, since Kim et al, reported an IC_{50} equal to 19.7 µM (33), Kempuraj et al found an inhibition of 36% at 100 µM (34) and Land et al, registered an IC_{50} > 32.0 µM (35). Whereas, found results present an IC_{50} less than 100 µM. via the LOX inhibition.

Evaluation of anti-acetyl-cholinesterase assay

The inhibitory effect on acetyl-cholinesterase have never been elucidated for M3, M13, M14, M15, M16, M17, M18 and M19. The half maximal inhibitory concentration IC_{50} of AChE was evaluated for 19 selected pure molecules (Table 2). Values of percentage of inhibition at 100 µM are as follows: 20.2 ± 5.5; 20.9 ± 4.7; 21.8 ± 5.0; 23.8 ± 4; 86.7 ± 1.1 for M7, M12, M13, M6, and M3, respectively. The remaining tested compounds had shown no inhibition effect on AChE activity. In the present study, founded results clearly showed that M3 was the most important compound with an interesting effect on AChE inhibition with an IC_{50} equivalent to 14.5 ± 1.0 µM. The chemical structure could be involved, actually, the presence of chlorine group for the 1, 4-naphthoquinone may lead to undergo the reaction with the acetyl cholinesterase and cause then its inhibition. As far as our knowledge could be certain, no study in the literature elaborated the anti- AChE activity of 2, 3-dichloro-5, 8-dihydroxy-1, 4-naphthoquinone. For the rest of compounds, other investigators had found an interesting effect on anti-Alzheimer activity via the aggregation of beta amyloid such as for M1, Kim et al. (36) who found an IC_{50} equivalent to 18.7 µM. Moreover, Sato et al. (37) showed an IC_{50} equal to 33, 15.1, 25.3 and 5.3 µM, for M4, M5, M8 and M10, respectively. However, Duan et al. (38) reported an IC_{50} equal to 24.5 ± 4.9 µM for M8 via the inhibition of AChE.

Table 2. *In vitro* anti-5-LOX and anti-AChE activities of tested molecules.

Founded results Anti-5-LOX		Anti-AChE
Compounds	**% inhibition (100 µM)**	
M1	5.9±7.7	na
M2	na	na
M3	na	86.7±1.1
M4	20.9±7.5	na
M5	65.3±4.3	na
M6	na	23.8±3.9
M7	4.7±4.4	20.2±5.5
M8	3.7±5.4	na
M9	na	na
M10	45.2±9.4	na
M11	52.9±4.9	na
M12	27.1±4.1	20.9±4.7
M13	37.5±4.7	21.7±5.0
M14	19.6±10.6	na
M15	22.6±10.3	na
M16	28.0±9.9	na
M17	28.4±8.9	na
M18	35.3±11.5	na
M19	26.3±0.4	na

Anti-α-glucosidase activity

Within selected compounds, there were some of them which have never been studied previously for the diabetic effect through the α-glucosidase inhibition assay, as M14, M15, M16, M17, M18 and M19. During this study, all molecules are found to have moderate inhibitory activity against α-glucosidase at a concentration of 100 µM, as shows Table 3. Since, there are several modes of action for a pure molecule to exert its effect, a single method seems not enough to confirm its potential. Therefore, those results does not confirm the disability of tested compounds to inhibit the α-glucosidase. Compared to others' work, obtained results are in line with those found by Shang et al. (39), who have registered no effect for M2 via inhibition of α-glucosidase. While, Nile and Park, (21), have found for M10 an IC_{50} equal to 211.5 µM via the inhibition of α-glucosidase. Furthermore, Dhanya et al. (40), have reported for M2, using another method based on monitoring a special fluorescent indicator for direct glucose uptake via 2NBDG (2-Deoxy-2-((7-nitro-2,1,3-benzoxadiazol-4-yl) amino)-D-glucose) in differentiated myoblasts, that it enhanced the fluorescence intensity of cells with 34.5% at 100 µM. Those results were supported by various *in vivo* investigators. As well, Ozcan et al. (41), have demonstrated that M5 could be a therapeutic agent in diabetic nephropathy, via the administration of M5 (6 mg/day) for streptozotocin induced diabetic in rats. Basing on the fact that diabetes is directly associated with lipid metabolism, Liang et al. (42), suggested the use of M5 as a lipid lowering drug, as it has hypolipidemic effect in hyperlipidemic mouse models at a dose of 0.5 g/kg. Moreover, Sun et al. (43) have reported that M4 could be a potential factor for the treatment of diabetic cardiomyopathy. Since, at a dose of 100 mg/kg, it was able to decrease the intracellular ROS level and inhibit cardiac

Table 3. Anti-α-glucosidase, anti XOD and anti-SOD activities of some compounds.

Obtained results	Anti-α-glucosidase	Anti-XOD	Anti-SOD	
Compounds	% Inhibition (100 µM)	%Inhibition (100 µM)	% Inhibition (100 µM)	IC_{50} (µM)
M1	na	na	102.8±9.4	36.0±2.0
M2	na	na	119.3±14.4	26.2±1.8
M3	43.8±5.6	na	99.2±11.9	10.0±0.0
M4	na	na	79.4±2.4	16.3±1.5
M5	33.2±7.5	na	82.1±8.1	41.9±3.5
M6	na	na	55.5±13.7	88.0±10.6
M7	na	na	65.6±13.8	78.5±2.3
M8	na	na	86.4±2.7	16.4±1.7
M9	23.9±8.4	na	103.3±3.8	37.9±0.1
M10	na	na	97.7±16.4	37.3±4.7
M11	na	na	82.8±6.8	16.2±1.8
M12	na	na	45.5±2.3	>100.0
M13	na	na	4.8±2.6	>100.0
M14	na	na	44.4±1.2	>100.0
M15	na	na	29.5±2.9	>100.0
M16	na	na	48.1±1.9	>100.0
M17	na	na	54.2±5.3	91.6±7.3
M18	na	na	19.7±9.7	>100.0
M19	na	46.5±5.1	68.0±2.4	47.7±6.8

"na": not active.

myocyte apoptosis. Also, Bouderba et al. (44), have found that M7, at a dose of 100 mg/kg, have interesting role, used alone or as an adjunct to anti-diabetic drugs, in the management of lipid disorders. For the same molecule, Guigas et al. (45), have suggested that M7 (100 µM) might be used as a therapeutic agent in treatment of type 2 diabetes, as it is an inhibitor of both hepatic glucose 6-phosphatase and gluconeogenesis. For M8, it has been reported by Shi et al. (46), that at a dose of 50 mg/kg, it has the ability to improve insulin sensitivity in skeletal muscle *in vivo*, so it could be used as an agent for the treatment of type 2 diabetes. Likewise, Zhou and Mao, (47), have registered that this compound can effectively improve the impaired glucose tolerance in animal model at a dose of 250 mg/kg. Concerning the M9, O¨berg et al. (48), have reported that it increases the glucose uptake in skeletal muscle cells for diabetic animals at a dose of 10 mg/kg. For the same molecule, Kamei et al. (49), have suggested that it could be used as a tool to treat diabetes, as it stimulates the glucose uptake via tyrosinase kinase dependent pathway, involving Akt phosphorylation (60 µM).

Anti-xanthine oxidase inhibitory activity

During this work, the anti-xanthine oxidase inhibitory activity of 19 pure flavonoids was examined. Some of them are evaluated for the first time for such purpose (M12, M13, M14, M15, M16, M17, M18 and M19). Results are presented on Table 3. The totality of selected compounds were found to have no inhibitory effect on the xanthine oxidase via this test (% inhibition= na), except M19, which has shown activity towards this enzyme (%inhibition= 46.5 ± 5.1). Unlike, Cimanga et al. (50), who have reported an inhibitory effect of M2, M5, and M10 with IC_{50} equal to 52.2 ± 0.4 µM; 2.4 ± 0.2 µM and 20.3 ± 1.7 µM, respectively. Furthermore, Dew et al. (51) have found for M2 and M10 an inhibitory effect against this enzyme with IC_{50} equivalent to 284 ± 55 µM and 63.4 ± 3.4 µM, respectively. Similarly, Nile and Park (21), have registered an interesting value for M10 of IC_{50} equal to 5.5 ± 0.4 µM via this enzymatic method. Also, Li et al. (9) have found that the M5 had an inhibitory effect towards XOD with an IC_{50} equal to 197.0 µM. Moreover, Hadj-Salem et al (52), have demonstrated that M10 did not display an interesting anti-XOD activity with an IC_{50} equivalent to 183 µM. It can be concluded that found results can't confirm for them alone the disabilities of tested molecules to inhibit the production of uric acid and to prevent the hyper-uricemia, as they act by different ways, it may be beneficial to evaluate this activity via other tests.

Effect on SOD activity

SOD is known, on one hand, as an antioxidant endogenous enzyme which is, on the other hand, involved in chemo resistance process of ovarian cancer cells. A possible mechanism by which bioflavonoids can be investigated for their implication in this phenomenon was explored by the effect of SOD on pyrogallol's autoxidation as mentioned previously, and their abilities of inhibiting this metabolic enzyme. Selected compounds have never been explored for such reason. During this work, the inhibitory effect of those products on SOD was evaluated, and for the first time, it was proven that most of the tested molecules induced inhibition of SOD activity, with IC_{50} values ranging from 10.0 ± 0.0 µM to 88.0 ± 10.6 µM for M3 and M6 respectively as shows Table 3. Compounds M3, M4, M8, and M11 have registered most interesting inhibitory abilities. This potent effect might be related to their chemical structure, actually, the presence of the aromatic ring with double oxygen bonded on $C_{4,}$ which linked with a ring with

$2H_3O$ and the hydroxyl group on C_5 enable the whole molecule to go through the reaction and to link to the bonded sites of enzyme and thus, inhibit its activity. Almost products that had anti-SOD activities are found in the present study as a source rich of antioxidants, thus, it may be explain the process by which these compounds react. Indeed, their ability of scavenging free radicals allows them to inhibit the SOD, consequently, to reduce chemo resistance of ovarian cancer cell lines.

Cell viability evaluation

The cell proliferation activity of tested compounds against human breast cancer cells MCF-7 and colorectal cancer HCT116 was assessed using the MTT assay, and presented in (Figure 1). It was clearly observed that the effect of pure compounds on MCF-7 breast cancer cell proliferation varied significantly among molecules. Compounds exhibited an interesting cytotoxic activity, unless M2, M4, M7, M10, M11, M13, and M15 (% inhibition < 67%) (Figure 1). Whereas, inhibition was more evident with M1, M3, M6, M9, M14, M16 and M17 (IC_{50} < 17.5 µM) (Table 1). For the colorectal cancer cell line HCT116, the majority of tested compounds had potential anti-cancer effect. They had an important IC_{50} values ranging between 0.2 ± 0.0, 0.3 ± 0.0, 0.4 ± 0.0, 1.5 ± 0.2, 9.0 ± 1.0, 14.0 ± 1.0, 17.0 ± 2.0 µM for M1, M3, M9, M14, M17, M18 and M16 respectively (Table 1). Other compounds had less anticancer abilities as M5, M6, M12, M10 and M8 representing moderate IC_{50} values of 28 ± 3; 27.0 ± 4.0; 32.0 ± 5.0; 74.0 ± 6.0 and 79.0 ± 7.0 µM, respectively. M1 showed the highest anticancer activity against HCT116 cell line (IC_{50} equal to 0.2 ± 0.0 µM) using MTT assay, followed by M3 with an IC_{50} equivalent to 0.3 ± 0.0 µM. Among compounds, M5, M6, M8, M9, M12, M14, M16, M17 and M18 showed interesting anti-proliferation activities against both cancer cell lines MCF7 and HCT116 as represented the Table 1.Since the literature pertaining to the anticancer activity of phenolic compounds is voluminous and it is reported that the existence of quinones allows some bioflavonoids to exert an anti-tumor effect (53), so it may be that the hydroxyl-naphthoquinone moiety endows M1, M3, M5, M6, M8, M9, M10, M12, M14, M16, M17, M18 and M19 with anti-carcinogenic abilities, anti-SOD effect and antioxidant activities proved in the present study, which may be also responsible for some of their pharmaceutical effects, such as anti-inflammatory effect (34) and anti-tumor (53). These results are in line with previous researches, with the involvement of antioxidants pathway in different human cancers (54). According to found results, it can be clearly seen that the HCT116 (12 from 19 molecules have cytotoxic activity) was less sensitive to the various tested products compared to MCF7 cell line (13 molecules from total of 19). Regarding to the value of IC_{50} ratio M1, M5, M9, M10, M12, and M18 exhibited a ratio close to 1, that reflect two hypothesis, either compounds are toxic, whether, efficient against two cell lines. For the rest of molecules; M3 and M14 presented a ratio bigger than 1, which reveals the selectivity of compounds towards HCT116 cancer cell line. While, M6, M8, M16, and M17 showed a ratio less than 1, and then could be more selective against MCF7 cell line. This phenomenon of selectivity of compounds towards various cancer cell lines may be

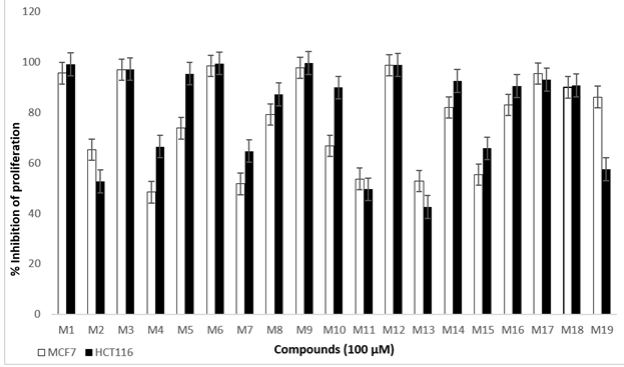


Figure 1. Anti-cancer effect of studied molecules toward breast cancer cells (MCF7) and colorectal cancer cell line (HCT116).

explained, by the involvement of their chemical structures as well as the cell's response behavior. Based on this ratio solely remains insufficient to sum up the toxicity index of products. Therefore, the evaluation of their cytotoxicity potential on healthy cells becomes an urgent necessity. Interestingly, the M9 anticancer activity against MCF7 cell line, herein reported is more interesting than what was described elsewhere. Actually, Seo et al. (55), via the resazurin reduction assay, have found that M9 has a cytotoxic effect against breast cancer cell line with an IC_{50} equal to 2.1 µM. Also, Baloch et al. (56), have found an IC_{50} equivalent to 1.8 µM via MTT test, against the same cancer cell line, as well as, Lin et al. (57), who reported for the same molecule, through the MTT assay, an IC_{50} equal to 0.56 µM, which is lower than what was found here (IC_{50}= 5 µM). Moreover, Kim et al. (58) have found an IC_{50} equal to 4.8 µM for M3 against MCF7 which is close to obtained result (3.6 ± 0.3 µM). In the same context, it has been reported that M3 is an important anti-tumor agent (59). Concerning M7, previous investigators have found cytotoxic effect towards MCF7 cell line. Since, Zhang et al. (60) have reported an IC_{50} (105 µM) threefold less important than the one herein described (30 µM). Whereas, Kellici et al. (61), have elucidated for the same molecule, using the MTT assay, a percentage of inhibition of 20% at 25 µM, which is a little bit higher than found with 50% at 100 µM. Unlike, obtained results for M10, that did not exhibited a cytotoxic effect against MCF7, previous works have proved its cytotoxic potential. Indeed, Yang and Liu (62), have found an IC_{50} of 46.4 µM against MCF7, and You et al. (63) reported a percentage of inhibition of 60% at 50 µM. Whereas, M12, which found to have an interesting percentage of inhibition during this work (98.8 % at 100 µM), was reported by to have no cytotoxic effect against MCF-7 cell line (<10 % at 25 mM) (64).

In the other hand, it has been reported by Amado et al. (65), that M10 has a moderate cytotoxic potential against HCT116 cell line, about 60% at 150 µM, which is less interesting than found results with 90% at a 100 µM (Figure 1). Whereas, You et al. (63) have proved for the same molecule, an 80% of inhibition against HCT116 was registered via the MTT test, at a concentration of 50 µM. Furthermore, for M12 EL-Naga (66) and Kim et al. (67) have found equal percentage of inhibition against HCT116 cell line equivalent to 80% at a concentration of 20 µM, and 25 µM, respectively. Those results are higher than what was found here with 99%

at 100 µM. These differences between obtained results and other investigators' may be explain, on one hand by using multiple distinct assays, and on the other hand, by the various pathways of bioflavonoids to exert their effect on different activities. To the best of our knowledge, this is the first study demonstrating that M16, M17, and M18 were explored for such purpose and could be then developed as a novel agent in the field of therapy.

During this work, different activities were evaluated for the first time for many molecules. Most of tested compounds with functional groups related to their basic aromatic rings, scavenge free radicals thus exhibiting an antioxidant activity. It has been shown that active oxygen species are implicated in cancer (54). Actually, in some steps of the anticancer process, the implication of free radicals is important, one of the strongest arguments in favor of this being the beneficial effect of superoxide dismutase (SOD) especially in joint cancer. Whereas, it is implicated on chemo resistance in ovarian cancer cells. The current study highlighted that the majority of investigated pure molecules are also potent SOD inhibitors**.** Thus, the above tested M1, M2, M5, M6, M8 and M9 appear to be effective inhibitors agents not only on breast cancer cells MCF7 but also on colorectal cancer cell line HCT116**.** Further works should be carried out using the most potential compounds to determine *in-vivo* antioxidant and anti-tumor activities, and to elucidate the exact mechanism involved in this compounds' biological activities. The present study provides evidence for the use of some traditional bioflavonoids. This may possibly be an opening to improve regular antioxidant and anticancer plants preparation from traditional knowledge and find out new natural units for the development of upcoming cancer and oxidative stress treatment. The majority of tested compounds was found to be significantly promising. This result suggests the need for more extensive evaluation of secondary metabolites for their remedial potential against different diseases.

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Declarations of interest

The authors report no declarations of interest.

Author's contribution

Imen Khelifi wrote the manuscript under the supervision of other authors. Validation of the experimental results was made by Jalloul Bouajila. El Akrem Hayouni, Sylvie Cazaux, Riadh Ksouri and Jalloul Bouajila refined and reviewed the draft for publication.

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